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Evidence that 5′-Cytidinediphosphocholine Can Affect Brain Phospholipid Composition by Increasing Choline and Cytidine Plasma Levels

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Abstract: We examined the effects of orally administered 5′-cytidinediphosphocholine (CDP-choline) on arterial plasma choline and cytidine levels and on brain phospholipid composition in rats. Animals receiving a single oral dose of 100, 250, or 500 mg/kg showed peak plasma choline levels 6–8 h after drug administration (from 12 ± 1 to 17 ± 2, 19 ± 2, and 24 ± 2 μM, respectively). The area under the plasma choline curve at >14 μM, i.e., at a concentration that induces a net influx of choline into the brain, was significantly correlated with CDP-choline dose. In rats receiving 500 mg/kg this area was 2.3 times that of animals consuming 250 mg/kg, which in turn was 1.8 times that of rats receiving 100 mg/kg. Plasma cytidine concentrations increased 5.4, 6.5, and 15.1 times baseline levels, respectively, 8 h after each of the three doses. When the oral CDP-choline treatment was prolonged for 42 and 90 days, brain phosphatidylcholine concentrations increased significantly (by 22–25%; $p < 0.05$) in rats consuming 500 mg/kg/day. Brain phosphatidylethanolamine and phosphatidylserine concentrations also increased significantly under some experimental conditions; levels of other phospholipids were unchanged. **Key Words:** Brain phospholipids—5′-Cytidinediphosphocholine—Choline—Cytidine—Phosphatidylcholine—Plasma levels.
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Although brain neurons can synthesize small amounts of choline, the bulk of brain choline derives from the circulation (Blusztajn and Wurtman, 1983). Any compound capable of causing a sufficient increase in blood choline levels might be expected also to affect brain choline, as the macromolecule that facilitates the bidirectional flux of choline across the blood–brain barrier is unsaturated at normal plasma choline concentrations (Cornford et al., 1978). Once inside the brain, choline disappears rapidly (Klein et al., 1990), principally by metabolism to phosphorylcholine (Millington and Wurtman, 1982) and then to phosphatidylcholine (PtdCho). In cholinergic neurons, much of the free choline is converted to acetylcholine (ACh). When such neurons are physiologically active they can also utilize choline derived from membrane PtdCho to synthesize the ACh (Maire and Wurtman, 1985). If free choline is in short supply, this process can lead to an actual decrease in the quantity of membrane, i.e., in the amounts of PtdCho and the other structural phospholipids per cell (Ulus et al., 1989). This process can be blocked by providing supplemental choline (Wurtman et al., 1990).

Orally administered 5′-cytidine diphosphocholine (CDP-choline), an endogenous intermediate in PtdCho biosynthesis (Kennedy and Weiss, 1956), is hydrolyzed before or during absorption to yield two major active metabolites: choline and cytidine (López G.-Coviella et al., 1987; Galletti et al., 1991). These compounds enter the bloodstream and are taken up into the brain and other tissues. As both choline and CTP (originating from cytidine) are substrates for PtdCho synthesis and as brain cytidine levels, like those of choline (Zeisel, 1981), are relatively low (López G.-Coviella et al., 1987), we investigated whether administering CDP-choline chronically could affect brain phospholipid levels. Preliminary results of this study have been presented (Agut et al., 1993).

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Abbreviations used: ACh, acetylcholine; AUC, area under the curve; CDP-choline, 5′-cytidinediphosphocholine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

MATERIALS AND METHODS

Female Sprague–Dawley rats weighing 200–250 g were housed in conventional cages and given free access to food (A04; U.A.R., France) and water; their food intake was monitored. The diet consisted of 17.2% protein, 2.7% fat, and 59.7% carbohydrate (the remaining being minerals, fiber, and water) and contained adequate amounts of vitamins and 1.58 mg/g of choline but no detectable CDP-choline. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Care Committee.

Plasma choline and cytidine levels

Groups of rats that had been previously cannulated in the left common carotid artery (PE-50 tubing; Medical Equipment Co.) received 100, 250, or 500 mg/kg of CDP-choline by gavage, following a 12-h fasting period. Blood samples were taken at zero-time (immediately before administration of the drug) and at 3, 6, 8, 10, and 12 h thereafter. Samples were centrifuged, the plasma specimens were deproteinized by adding 0.4 *M* perchloric acid and centrifuged again, and the supernatants were lyophilized and stored at −70°C until assay.

Plasma choline content was determined by HPLC, using a polymeric reversed-phase column (BAS, West Lafayette, IN, U.S.A.), coupled to an electrochemical detector (model 200a; BAS). An enzymatic postcolumn reactor containing choline oxidase (EC 1.1.3.17), covalently attached to the postcolumn base, converted choline to hydrogen peroxide, which is electrochemically detected by a platinum electrode at 500 mV. The mobile phase consisted of 50 m*M* sodium phosphate (pH 8.5), containing 0.005% Kathon CG as a bactericide.

Nucleosides were purified by boronate-affinity gel chromatography (Affi-gel 601; Bio-Rad Laboratories, Richmond, CA, U.S.A.) before analysis by HPLC. To separate the ribonucleoside fractions, dried residues of the samples were redissolved in 0.25 *M* ammonium acetate (pH 8.8) and applied at 4°C to gel columns (1.0 × 1.5 cm) that had been equilibrated with 0.25 *M* ammonium acetate (pH 8.8). The columns were washed twice with 5 ml of the ammonium acetate solution, and the ribonucleosides were eluted with 5 ml of 0.1 *M* formic acid. Fractions containing ribonucleosides were dried under vacuum and redissolved in water (100 μl) just before analysis by HPLC.

Cytidine concentrations were measured by HPLC and UV detection (at a wavelength of 280 nm) using a reverse-phase column (Dynamax; 250 × 4.6 mm; ODS 3 μm) with a gradient system—solvent A, 10 m*M* KH_2PO_4 and 1% methanol, pH 6; solvent B, 50% methanol in solvent A, pH 6; 5-min linear gradient to 50% solvent B (WISP controller; Waters Associates, Bedford, MA, U.S.A.) followed by 5 min of 100% solvent B; flow rate, 0.7 ml/min, with a 15-min equilibration delay.

Brain phospholipids

Twelve-month-old rats were divided into 10 groups. One group received 500 mg/kg/day of CDP-choline by gavage for 21 days, and a control group received the same volume of vehicle alone. Six more groups received 100, 250, or 500 mg/kg/day of CDP-choline in the diet, for 42 and 90 days. The remaining two groups of animals were used as controls (they consumed the diet with vehicle) for these two treatment periods. At the end of each treatment period (when animals were 12 months and 21 days, 13 months and 12 days, and 15 months of age), the animals were killed at noon by decapitation under light anesthesia (Pentothal; 25 mg/kg, i.p.); their heads were dropped into liquid nitrogen and stored at −70°C for subsequent phospholipid analyses.

Brain phospholipids were extracted according to the method of Folch et al. (1957). Brain tissue, corresponding to frontoparietal cortex, was dissected, frozen on dry ice, sonicated in methanol, brought to 20% (vol/vol) with chloroform/methanol (2:1 vol/vol), and mixed with 2 volumes of a 50% methanol/water solution. After centrifugation the organic phase (locus of the phospholipids) was dried under vacuum. The residue was reconstituted in chloroform/methanol (1:1 vol/vol), and an aliquot (20 μl) of the phospholipid extract was subsequently purified by TLC on silica gel G plates (LK-6D or LK-5D plates; Whatman Co.), using a system consisting of chloroform/ethanol/triethylamine/water (30:34:30:8 by volume) as the mobile phase. Phospholipid standards were used to identify the corresponding bands under UV light after the plates were sprayed with 0.1% diphenylhexatriene in petroleum ether. The total amounts of each of the phospholipids were determined by phosphate assay (Svanborg and Svennerholm, 1961) and expressed per milligram of protein. Total protein levels were determined from tissue extract interphases after sonication in 0.1 *M* NaOH, using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

Data analysis

Statistical analysis of the data was performed by one or two-way ANOVA and post hoc comparisons between means (Tukey-Kramer test; using statistical software by Systat, Evanston, IL, U.S.A.). Data on plasma choline and cytidine levels were used to calculate the areas under the curve (AUCs) for the three doses of CDP-choline used. After transformation of the data into straight lines (by representing the AUC in semilogarithmic plots), correlation analyses of the AUC versus CDP-choline dose were done using linear regression models. The number of animals per group used for brain phospholipid analyses varied between eight and 19 and were five for plasma choline and cytidine level determinations. Results represent the AUC or mean ± SEM values of at least two separate experiments.

RESULTS

Effects of acute CDP-choline administration

To quantify the changes in plasma choline and cytidine levels that occur after a single oral dose of CDP-choline, rats previously fasted for 12 h received 100, 250, or 500 mg/kg of the drug (Fig. 1). Six hours later, plasma choline levels had increased by 42 (from 12 ± 0.5 to 17 ± 2 μ*M*), 58 (from 12 ± 1 to 19 ± 2 μ*M*), or 100% (from 12 ± 1 to 24 ± 2 μ*M*), respectively ($p < 0.01$). These levels remained high for an additional 2 h, still not returning to baseline by the time of the last measurement (12 h after administration of the drug), i.e., they were still increased by 17, 25, and 33%, respectively.

Figure 2A represents these results as the semilogarithmic plots of the AUCs and shows the correlations

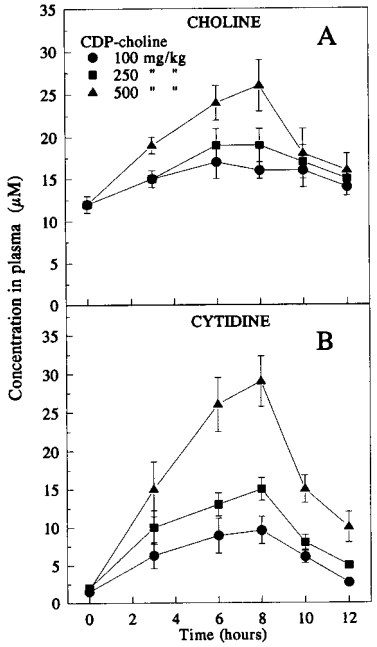

FIG. 1. Effects of various doses of orally administered CDP-choline on plasma (**A**) choline and (**B**) cytidine levels. Groups of five rats that had been previously cannulated in the left common carotid artery received 100, 250, or 500 mg/kg of CDP-choline by gavage, following a 12-h fasting period. Blood samples were taken immediately before administration of the drug and at 3, 6, 8, 10, and 12 h thereafter. Plasma choline and cytidine levels were measured as described in Materials and Methods. Data are mean ± SEM (bars) values.

between CDP-choline dose and the plasma choline AUC ($r^2 = 0.9885$). We also calculated the AUCs for plasma choline concentrations of >14 μM [because choline flux is from blood to brain at and above this concentration (Klein et al., 1990)] and correlated them with the three doses used (Fig. 2B). This correlation was also highly significant ($r^2 = 0.9987$; $p < 0.01$): The AUC in rats receiving 500 mg/kg of CDP-choline was 2.3 times that of animals receiving 250 mg/kg, which in turn was 1.8 times that of rats receiving 100 mg/kg.

Fasting plasma cytidine levels were between 1.5 and 2 μM and rose to 6.3 ± 1.7, 10 ± 2.0, and 15 ± 3.6 μM, respectively, 3 h after administration of 100, 250, or 500 mg/kg of CDP-choline ($p < 0.01$; Fig. 1B). Peak cytidine levels (5.4, 6.5, and 15.1 times over baseline, respectively) were attained 8 h after CDP-choline administration. Plasma cytidine content remained elevated in arterial blood for at least an additional 4 h (2.7 ± 0.2, 5 ± 0.4, and 10 ± 2 mM, respectively). The AUC for plasma cytidine levels also correlated significantly with the CDP-choline dose ($r^2 = 0.9970$; Fig. 2C). The AUC in rats receiving 500 mg/kg of CDP-choline was 1.8 times that of animals receiving 250 mg/kg, which in turn was 1.5 that of rats receiving 100 mg/kg.

Brain phospholipid content

To determine whether long-term elevations in plasma choline and cytidine levels could affect brain phospholipid content, groups of rats received 500 mg/kg/day of CDP-choline for 21 days and 100, 250, or 500 mg/kg/day, with the diet, for 42 and 90 days.

Administration of 500 mg/kg/day of CDP-choline for 21 days did not significantly alter total brain phospholipid content, or those of cortical PtdCho, phosphatidylethanolamine (PtdEtn), or phosphatidylserine (PtdSer) (Fig. 3). Increasing the length of treatment to 42 (Fig. 4A) or 90 days (Fig. 4B) caused significant elevations in cortical PtdCho, PtdEtn, and PtdSer concentrations but not in levels of other phospholipids measured (phosphatidylinositol, phosphatidic acid, and sphingomyelin; data not shown). After 42 days of CDP-choline administration, PtdCho content increased by 19% in rats receiving 100 mg/kg/day ($p < 0.05$) and by 22% in rats receiving 500 mg/kg/day. PtdEtn and PtdSer concentrations were increased by 17 (p

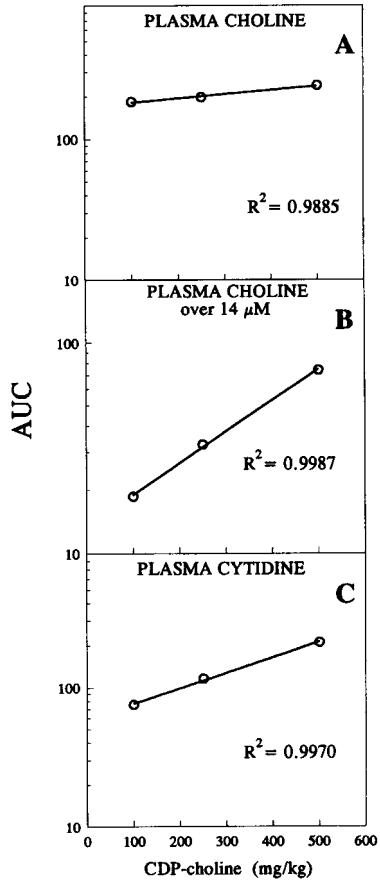

FIG. 2. A–C: Semilogarithmic representation of the effect of a single oral dose of CDP-choline on plasma choline and cytidine levels, expressed as the AUC. For details, see the legend to Fig. 1. Symbols represent the AUC for each of the three doses over a period of 12 h. r^2 is the coefficient of determination from the linear regression analysis.

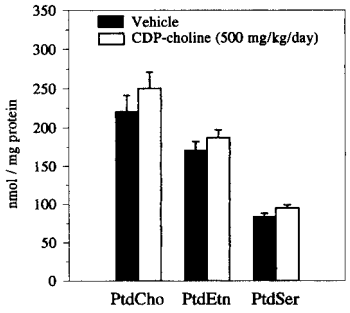

FIG. 3. Effect of administering CDP-choline for 21 days on PtdCho, PtdEtn, and PtdSer concentrations in frontoparietal cortex of 12-month-old rats. Groups of rats received CDP-choline (500 mg/kg/day, by gavage) or vehicle for 21 days and were killed. Levels of brain phospholipids were measured as described in Materials and Methods. Data are mean ± SEM (bars) values.

< 0.05) and 42% ($p < 0.01$), respectively. Among rats receiving 500 mg/kg/day for 90 days, PtdCho content increased by 25% ($p < 0.01$), PtdEtn by 17% ($p < 0.05$), and PtdSer by 42% ($p < 0.01$). No significant changes were observed with the other two doses used.

DISCUSSION

These data show that CDP-choline, in doses that increase plasma choline and cytidine levels, can produce dose- and time-related increases in brain levels of PtdCho and other phospholipids. High doses of CDP-choline maintain plasma choline levels at >14 μM for longer periods (Figs. 1 and 2) and hence may substantially increase the net uptake of choline into the brain (Klein et al., 1990), which is necessary for PtdCho synthesis. When lower doses of CDP-choline are used, these increments in brain PtdCho levels are more variable, not always significant or dose-dependent, and perhaps more dependent on factors such as the animal's eating pattern, the absorption and metabolism of the drug, and the blood–brain barrier transport of its metabolites.

Among fasting rats, in which arterial plasma choline levels oscillate between 5 and 12 μM (Zeisel, 1981), the choline concentrations in the venous cerebral outflow are usually higher than in arterial blood entering the brain, suggesting a net release of this compound from brain "reservoirs" (Aquilonius et al., 1975; Tuček, 1978; Klein et al., 1990). However, if the choline concentration of the arterial blood is increased to ≥14 μM, this condition can be reversed, causing a net influx of choline into the brain (Klein et al., 1990). Within 6 h of receiving a single oral dose of 100, 250, or 500 mg/kg CDP-choline, plasma choline levels increased from 12 to 17, 19, or 24 μM, respectively. The total amounts of choline administered were equivalent to 35, 88, or 175 mg/kg, respectively, and the resulting increases in plasma choline concentrations were similar to those found by other investigators using other choline sources, such as choline chloride (Haubrich et al., 1976) or PtdCho (Hirsch et al., 1978; Zeisel, 1981). Because choline is carried into and out of the brain by a bidirectional transport system (Cornford et al., 1978), which depends on arteriovenous differences in plasma choline, its brain uptake could be enhanced if CDP-choline administration caused a sufficient rise in plasma choline levels. The AUC at >14 μM plasma choline (over a 12-h period) in rats receiving a single dose of 500 mg/kg of CDP-choline was more than twice that of rats treated with 250 mg/kg and about four times that of rats receiving 100 mg/kg. By increasing arterial choline concentrations above this level (14 μM) administration of CDP-choline should enhance the influx of choline into the brain, where it would rapidly be converted to phosphorylcholine (Millington and Wurtman, 1982), a precursor of brain PtdCho, as well as ACh (Maire and Wurtman, 1985).

The administration of CDP-choline not only increased plasma choline levels, but also those of cytidine in arterial blood. Plasma cytidine concentrations rose severalfold 8 h after administration of a single drug dose (100, 250, or 500 mg/kg). Although a transport system for the uptake of cytidine into the brain has not been fully characterized, measurements of the

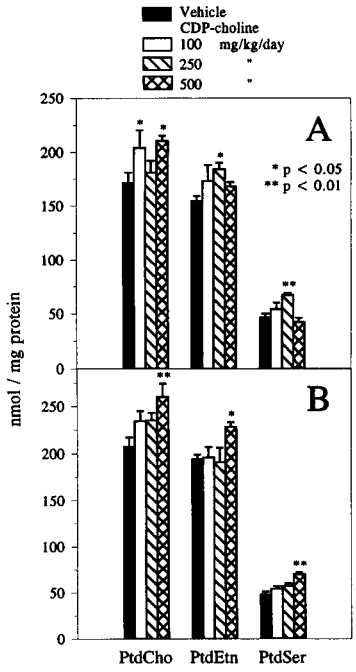

FIG. 4. Effect of administering CDP-choline for 42 and 90 days on PtdCho, PtdEtn, and PtdSer concentrations in frontoparietal cortex of 12-month-old rats. Groups of rats received CDP-choline (100, 250, and 500 mg/kg/day, incorporated into the diet) or vehicle for 42 (**A**) and 90 (**B**) days and were killed. Levels of brain phospholipids were measured as described in Materials and Methods. Data are mean ± SEM (bars) values.

arteriovenous differences in plasma cytidine concentrations occurring during a constant intravenous cytidine infusion suggest that >30% of the circulating cytidine enters the brain during a single pass (authors' unpublished data). Within cells, cytidine is sequentially phosphorylated to CTP, which could be a critical regulator of PtdCho synthesis (Choy et al., 1980; Vance et al., 1980). The combination of CTP with phosphorylcholine (brain levels of which would also be elevated, because of the increase in plasma choline levels after CDP-choline administration) would generate intracellular CDP-choline, itself a rate-limiting substrate in PtdCho biosynthesis (Vance et al., 1980; Whitehead et al., 1981; López G.-Coviella and Wurtman, 1992). Thus, we anticipated that long-term administration of adequate doses of CDP-choline would increase brain PtdCho content—a hypothesis compatible with our present findings.

In our experience, rat brain phospholipid levels can vary among groups of animals, depending on, among other things, their age, weight, sex, and type of diet. In the present experiments, we controlled for these and other variables on a daily basis and included a control group pair-fed to animals receiving the vehicle for each of the groups treated with CDP-choline. Thus, variations in brain phospholipid levels should have been a consequence of CDP-choline intake and not differences in age, i.e., 12 months and 21 days, 13 months and 12 days, and 15 months of age, weight, or amount of food ingested.

Rats receiving CDP-choline via various treatment regimens did exhibit significantly higher levels of PtdCho and other related phospholipids, like PtdEtn and PtdSer, than untreated animals. Elevations in PtdCho content were maximal (25% above controls) when rats received 500 mg/kg/day, the highest dose, for 90 days, the longest period tested. However, the elevations in brain PtdCho levels between groups of rats treated with 500 mg/kg/day for 42 and 90 days were similar. The reasons for this are not obvious; perhaps there is a limited range of brain PtdCho levels within which variations can be observed. Parallel increments in the concentrations of two other phospholipids, PtdEtn and PtdSer, were also observed with certain treatment regimens, i.e., in rats treated during 42 days with 250 mg/kg/day of CDP-choline. PtdCho, PtdEtn, and PtdSer are metabolically related phospholipids; the increase in level of one of them may lead, in some cases, to compensatory elevations in levels of the other two (Slack et al., 1989; López G.-Coviella and Wurtman, 1992). However, the nature of such compensatory mechanisms is unknown, and we are unable to explain why some groups of rats receiving CDP-choline for 42 and 90 days failed to show increased brain phospholipid levels.

The significance of the present results lies in the fact that choline, a metabolically expensive substrate, can undergo either of two fates in cholinergic neurons: It can be phosphorylated to phosphorylcholine by choline kinase and converted into PtdCho (as in other cells), or it can be acetylated to form ACh by choline acetyltransferase. These two enzymes exhibit low affinities for choline; hence, the actual rates of phosphorylcholine and ACh synthesis can depend on the concentration of choline available (Haubrich et al., 1975; Cohen and Wurtman, 1976). When cholinergic neurons are physiologically active and choline is in short supply, most of it will be funneled into ACh synthesis (Maire and Wurtman, 1985), thus sustaining neurotransmission at the expense of membrane building. If this situation persists, cell membranes will be consumed (Wurtman et al., 1990). Supplementation with choline, in concentrations in the high normal range, can block this reduction in phosphatide content while maintaining cholinergic neurotransmission (Ulus et al., 1989). Moreover, concurrent administration of cytidine in the form of CDP-choline can further accelerate phospholipid membrane formation (López G.-Coviella and Wurtman, 1992). Such a mechanism could underlie the therapeutic use of this drug (Zappia et al., 1985).

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